

**ISOLATION AND EXPRESSION OF HEMOLYSIN E (HlyE)
FROM *Salmonella enterica* serovar Typhi (*S. Typhi*) ISOLATES**

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FROM *Salmonella enterica* serovar Typhi (S. Typhi) ISOLATES**

by

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TABLE OF CONTENTS

ACKNOWLEDGMENT	ii
TABLE OF CONTENTS	iv
LIST OF TABLES	x
LIST OF FIGURES	xi
LIST OF SYMBOLS AND ABBREVIATIONS	xiii
ABSTRAK	xiv
ABSTRACT	xvi
CHAPTER 1	18
INTRODUCTION	18
1.1 <i>Salmonella</i> and disease: An overview	18
1.2 Typhoid fever.....	19
1.3 <i>Salmonella enterica</i> serovar Typhi	20
1.4 Laboratory diagnosis of typhoid fever	23
1.4.1 Identification of <i>S. Typhi</i> by culture method.....	23
1.4.2 Serological diagnostic methods	23
1.5 Pathogenicity of <i>S. Typhi</i>	24
1.6 <i>Salmonella</i> Pathogenecity Island (SPI).....	26
1.7 Function of HlyE in <i>S. Typhi</i> pathogenesis	28
1.8 Periplasmic protein releasable by osmotic shock	30
1.9 Rationale of study	32
1.9.1 General objective	35
1.9.2 Research objectives	35
CHAPTER 2	37
MATERIALS AND METHODS	37
2.1 Materials	37
2.1.1 Bacterial isolates.....	37
2.1.2 Cloning and expression vector	39
2.1.3 Sera samples	39
2.1.4 U937 human monocytic cell line.....	39
2.2 Media and chemicals.....	39
2.2.1 Media preparation for culture purposes.....	42
2.2.1 (a) Nutrient broth	42

2.2.1 (b) Nutrient agar	42
2.2.1 (c) Luria Bertani (LB) broth.....	42
2.2.1 (d) LB agar	42
2.2.1 (e) LB agar with ampicillin or kanamycin	43
2.2.1 (f) Salmonella Shigella (SS) agar	43
2.2.1 (g) Blood agar.....	43
2.2.1 (h) Triple Sugar Iron (TSI) agar	43
2.2.1 (i) Urea agar base	43
2.2.1 (j) Simmons citrate agar	44
2.2.1 (k) Methyl Red Vogas-Proskauer (MRVP) medium.....	44
2.2.1 (l) Sulphate Indole Motility (SIM) medium	44
2.2.1 (m) Sodium hydroxide (NaOH) (3.0 M)	45
2.2.2 Buffer for isolation of periplasmic protein	45
2.2.2 (a) 20 mM Tris-HCl, pH 8.0	45
2.2.2 (b) Resuspend buffer, pH 8.0	45
2.2.3 Buffer for cloning and expression	45
2.2.3 (a) Agarose 1% (w/v).....	45
2.2.3 (b) Cell lysis buffer (7 M urea, 20 mM HEPES, pH 7.0).....	46
2.2.3 (c) 1M IPTG stock solution	46
2.2.3 (d) Tris (1.0 M).....	46
2.2.3 (e) Ethylenediaminetetracetic Acid (EDTA) (0.5 M)	46
2.2.3 (f) Tris EDTA (TE) buffer (10 mM Tris, 1 mM EDTA).....	47
2.2.3 (g) Phosphate buffered saline (PBS) (1X, pH 7.4).....	47
2.2.3 (h) Ampicillin stock solution (100 mg/mL)	47
2.2.3 (i) Kanamycin stock solution (50 mg/mL)	47
2.2.3 (j) 100 mM Magnesium Chloride (MgCl ₂)	47
2.2.3 (k) 100 mM Calcium Chloride (CaCl ₂).....	48
2.2.4 Buffer preparation for SDS-PAGE and Western blot	48
2.2.4 (a) Resolving gel buffer, pH 9.3	48
2.2.4 (b) Stacking gel buffer, pH 6.8.....	48
2.2.4 (c) Ammonium persulfate (AP) 20%	49
2.2.4 (d) Sample buffer, pH 6.8.....	49
2.2.4 (e) Running buffer.....	49
2.2.4 (f) Coomassie blue stain	49
2.2.4 (g) Coomassie destaining solution	49

2.2.4 (h) Western blot transfer buffer.....	50
2.2.4 (i) 3% blocking solution	50
2.2.5 Preparation of reagents for histadine-tagged protein purification.....	50
2.2.5 (a) Binding buffer.....	50
2.2.5 (b) Washing buffer	50
2.2.5 (c) Elution buffer.....	51
2.2.6 Buffer and media preparation for cell culture	51
2.2.6 (a) Phosphate buffered saline (PBS) (1X, pH 7.2).....	51
2.2.6 (b) Complete media.....	51
2.3 Methods.....	52
2.3.1 DNA extraction and PCR amplifications	52
2.3.1.1 Reconfirmation of isolates of <i>S. Typhi</i> from the glycerol stock	52
2.3.1.2 Genomic DNA extraction	52
2.3.1.3 Agarose gel electrophoresis	53
2.3.1.4 PCR screening for <i>aroC</i> and <i>hlyE</i> of <i>S. Typhi</i> isolates.....	54
2.3.1.5 DNA purification from PCR product and enzyme reaction mixtures	54
2.3.1.6 Reconfirmation of <i>hlyE</i> by small fragments PCR and sequencing	55
2.3.2 Assays for hemolysin activity.....	58
2.3.2.1 Haemolytic display on blood agar	58
2.3.2.2 Disk-diffusion assay on blood agar	58
2.3.3 Isolation of periplasmic protein by osmotic shock.....	59
2.3.4 Construction of recombinant plasmid.....	60
2.3.4.1 Amplication of <i>hlyE</i> by PCR for cloning.....	60
2.3.4.2 Gel purification of DNA	62
2.3.4.3 Restriction enzyme digestion.....	62
2.3.4.4 Preparation of competent BL21 <i>E. coli</i> cells	63
2.3.4.5 <i>hlyE</i> cloned into pCR2.1-TOPO vector	64
2.3.4.6 Ligation.....	65
2.3.4.7 Transformation of competent cell.....	65
2.3.4.8 Plasmid extraction.....	66
2.3.4.9 Analysis of recombinant plasmid	67
2.3.4.9.1 PCR colony screening	67

2.3.4.9.2	Restriction endonuclease digestion of DNA	68
2.3.4.9.3	DNA sequencing	68
2.3.5	Expression of the <i>hlyE</i> gene	68
2.3.5.1	Induction of <i>hlyE</i> with IPTG	68
2.3.5.2	Preparation of lysed cells	69
2.3.5.3	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).....	70
2.3.5.4	Peptide analyzing by MALDI-TOF/TOF mass spectrometry	71
2.3.5.5	Western blot	72
2.3.6	Protein purification	73
2.3.6.1	Preparation of samples for protein purification	73
2.3.6.2	Immobilized metal affinity chromatography (IMAC)	74
2.3.6.3	Purification of histidine-tagged protein by immobilized Ni ²⁺ absorption chromatography	75
2.3.7	Cytotoxicity assays of mammalian cells	75
2.3.7.1	Thawing of cells from frozen storage	75
2.3.7.2	Sub-culturing of the cells	76
2.3.7.3	Cell counting.....	76
2.3.7.4	Cryo-storage of the cells	77
2.3.7.5	Seeding of cell and cytotoxicity test	77
2.3.7.6	Cell viability and 50% inhibitory concentration (IC ₅₀)	78
CHAPTER 3	80
EXPERIMENTAL DESIGN AND RESULTS	80
3.1	PCR screening for <i>aroC</i> and <i>hlyE</i> genes in clinical isolates of <i>S. Typhi</i>	80
3.1.1	Screening for <i>aroC</i> gene by PCR	80
3.1.2	Screening for <i>hlyE</i> gene by PCR	82
3.1.3	Confirmation of <i>hlyE</i> PCR product by sequencing	82
3.2	Hemolysis activity of <i>S. Typhi</i> isolates.....	86
3.2.1	Haemolytic display on blood agar	86
3.2.2	Confirmation assay for hemolysis by a modified disk-diffusion assay on blood agar.....	86
3.2.3	SDS-PAGE analysis of periplasmic protein (crude protein)	89
3.2.4	Haemolytic activity of crude periplasmic protein on blood agar	91
3.2.5	Western blot analysis of periplasmic protein against typhoid sera.....	93
3.2.6	Protein identification (periplasmic proteins) by mass spectrometry	95

3.3	Production of recombinant plasmid	105
3.3.1	<i>hlyE</i> cloned into pCR2.1-TOPO (intermediate vector)	105
3.3.1.1	Amplification of <i>hlyE</i> by PCR.....	105
3.3.1.2	PCR screening of positive clones (pCR2.1-TOPO construct)...	107
3.3.1.3	Restriction enzyme digestion of positive clones (pCR2.1-TOPO construct)	107
3.3.1.4	Confirmation of pCR2.1-TOPO construct by sequencing.....	110
3.3.2	<i>hlyE</i> cloned into pET50b (expression vector)	113
3.3.2.1	PCR screening of positive clones (pET50b construct)	113
3.3.2.2	Restriction enzyme digestion of positive clones (pET _{hly} T2)....	114
3.3.2.3	Confirmation of pET _{hly} T2 construct by sequencing	114
3.4	Expression of HlyE recombinant protein.....	118
3.4.1	Verification of target recombinant protein by SDS-PAGE analysis	118
3.4.2	Optimization for the expression of the HlyE recombinant protein.....	120
3.4.2.1	Induction of the HlyE recombinant protein at different intervals.....	120
3.4.2.2	Induction of the recombinant protein with different incubation temperatures	122
3.4.3	Determination of immunoreactivity of the expressed recombinant protein by Western blot	124
3.4.4	Protein identification (HlyE recombinant protein) by mass spectrometry	125
3.5	HlyE recombinant protein purification	131
3.5.1	SDS-PAGE analysis of fractions collected by performing IMAC technique	131
3.5.2	Verification of fractions collected by Western blot.....	132
3.6	Cytotoxicity effects of purified HlyE recombinant protein	135
3.6.1	24 hours incubation of treatment	135
3.6.2	48 hours incubation of treatment	137
CHAPTER 4		139
DISCUSSION		139
4.1	Presence of intact <i>hlyE</i> gene in <i>S. Typhi</i> vaccine strain Ty21a and <i>S. Typhi</i> isolates	139
4.2	Detection of haemolytic activity of <i>S. Typhi</i> isolates using ampicillin-disk diffusion assay	140
4.3	Presence of haemolytic activity in periplasmic proteins of <i>S. Typhi</i> vaccine strain and clinical isolate	142

4.4	Expression of HlyE recombinant protein in <i>E. coli</i> system	144
4.5	Purification of HlyE recombinant protein using IMAC	148
4.6	Cytotoxicity effects of HlyE recombinant protein on U937 human monocytic cell	149
CHAPTER 5		150
CONCLUSION AND RECOMMENDATION FOR FUTURE RESEARCH.....		150
5.1	Recommendation for future research.....	151
REFERENCES.....		152
LIST OF ABSTRACT PUBLICATIONS		160

LIST OF TABLES

Table 1.1:	Biochemical tests for differentiation of <i>S. Typhi</i> from related subspecies (adapted from WHO, 2003).....	22
Table 2.1:	<i>E. coli</i> strains used for cloning and expression study.....	38
Table 2.2:	List of chemicals, reagents and media used in this study.....	40
Table 2.3:	List of primers	56
Table 3.1:	Total number of <i>S. Typhi</i> isolates that harbored <i>hlyE</i> and displayed hemolysis on blood agar by ampicillin-disk diffusion assay	88
Table 3.2:	Details of the Mascot analysis for 55 kDa protein band from <i>S. Typhimurium</i>. 55 kDa proteins of <i>S. Typhimurium</i> were analysed by MALDI-TOF/TOF. Mass spectra were analysed to identify protein(s) of interest using Mascot sequence matching software with Ludwig NR database. Proteins score greater than 84 is significant ($p < 0.05$).....	97
Table 3.3:	Matched peptides against fliC Phase 1-l flagellin <i>Salmonella Typhimurium</i> amino acid sequence.....	99
Table 3.4:	Details of the Mascot analysis for 60 kDa protein band from <i>S. Typhi</i>. 60 kDa proteins of <i>S. Typhi</i> were analysed by MALDI-TOF/TOF. Mass spectra were analysed to identify protein(s) of interest using Mascot sequence matching software with Ludwig NR database. Proteins score greater than 84 is significant ($p < 0.05$)	101
Table 3.5:	Matched peptides against fliC Phase 1-l flagellin <i>Salmonella Typhi</i> amino acid sequence	103
Table 3.6:	Details of the Mascot analysis for recombinant protein. ~74 kDa proteins were analysed by MALDI-TOF/TOF. Mass spectra were analysed to identify protein(s) of interest using Mascot sequence matching software with Ludwig NR database. Proteins score greater than 85 is significant ($p < 0.05$)	127
Table 3.7:	Matched peptides against transcription elongation protein nusA <i>Escherichia coli</i> amino acid sequence.....	129
Table 3.8:	Raw data, mean value and percentage of cell viability for 24 hours incubation of treatment.....	136
Table 3.9:	Raw data, mean value and percentage of cell viability for 48 hours incubation of treatment.....	138

LIST OF FIGURES

Figure 1.1:	Flow chart of methodology	36
Figure 2.1:	Schematic diagram of small fragments for <i>hlyE</i>	57
Figure 2.2:	Location of sense and antisense primers in the <i>hlyE</i> gene coding sequence designed for cloning purposes	61
Figure 3.1:	PCR product of <i>aroC</i> gene amplification resolved on 1% agarose gel electrophoresis.....	81
Figure 3.2:	PCR product of <i>hlyE</i> resolved on 1% agarose gel electrophoresis.....	83
Figure 3.3:	Alignment of <i>hlyE</i> sequence between the vaccine strain Ty21a, two <i>S. Typhi</i> clinical isolates and <i>S. Typhi</i> CT18 (reference sequence) using BioEdit software	85
Figure 3.4:	Hemolysis by <i>S. Typhi</i> on the blood agar plate.....	87
Figure 3.5:	SDS-PAGE analysis of periplasmic protein (crude protein).....	90
Figure 3.6:	Haemolytic activity of crude proteins (periplasmic proteins) on blood agar	92
Figure 3.7:	Western blot analysis of crude protein (periplasmic protein)	94
Figure 3.8:	Amino acid sequence of Phase 1-I flagellin of <i>S. Typhimurium</i> . The underlined amino acids were the peptides identified in MALDI-TOF/TOF mass spectrometry of 55 kDa protein band from SDS-PAGE	100
Figure 3.9:	Amino acid sequence of Phase 1-I flagellin (<i>Salmonella Typhi</i>). The underlined amino acids were the peptides identified in MALDI-TOF/TOF mass spectrometry of 60 kDa protein band from SDS-PAGE	104
Figure 3.10:	PCR product of <i>hlyE</i> gene resolved on 1% agarose gel electrophoresis.....	106
Figure 3.11:	PCR screening of positive clones (pCR2.1-TOPO construct) resolved on 1% agarose gel electrophoresis.....	108
Figure 3.12:	Restriction enzyme analysis of positive clones (pCR2.1-TOPO construct) resolved on 1% agarose gel electrophoresis.....	109
Figure 3.13:	Alignment of recombinant pCR2.1-TOPO (clone 2) sequence with <i>S. Typhi</i> CT18 as reference sequence using BioEdit software	112
Figure 3.14:	PCR screening of positive clones (pET50b construct) resolved on 1% agarose gel electrophoresis	115
Figure 3.15:	Restriction enzyme analysis of positive clones (pET _{hly} T2) resolved on 1% agarose gel electrophoresis.....	116
Figure 3.16:	Sequence of pET _{hly} T2 recombinant vector	117

Figure 3.17: SDS-PAGE analysis of HlyE recombinant protein	119
Figure 3.18: SDS-PAGE analysis of recombinant pETHlyT2 at several induction intervals	121
Figure 3.19: SDS-PAGE analysis of recombinant pETHlyT2 induced at different incubation temperatures.....	123
Figure 3.20: Western blot analysis of the expressed HlyE recombinant protein.....	126
Figure 3.21: An amino acid sequence of NusA <i>Escherichia coli</i> Peptides identified from Mascot search engine against NusA are underlined.....	130
Figure 3.22: SDS-PAGE analysis of the fractions collected by performing IMAC.....	133
Figure 3.23: Western blot analysis of the fractions collected from IMAC.....	134
Figure 3.24: Cytotoxicity effects of HlyE recombinant protein on U937 cell line at 24 hours	136
Figure 3.25: Cytotoxicity effects of HlyE recombinant protein on U937 cell line at 48 hours	138

LIST OF SYMBOLS AND ABBREVIATIONS

AP	Alkaline persulfate
bp	Base pair
dH ₂ O	Distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMAC	Immobilized metal affinity chromatography
IPTG	Isopropyl-beta-D-thiogalactopyranoside
kb	Kilobase
kDa	KiloDalton
L	Liter
mA	Milliampere
mg	Milligram
mL	Milliliter
MW	Molecular Weight
nm	Nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
pH	Potential Hydrogeni
RE	Restriction enzyme
rpm	Revolutions per minute
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SPI	Salmonella Pathogenecity Island
<i>Taq</i> DNA polymerase	<i>Thermus aquaticus</i> DNA polymerase
TEMED	N, N, N', N'- tetramethylethylenediamine
UV	Ultraviolet
V	Voltage
μL	Microliter
μg	Microgram

**PENGASINGAN DAN PENGEKSPRESAN HEMOLISIN E (HlyE)
DARIPADA *Salmonella enterica* serovar Typhi (*S. Typhi*) ISOLAT**

ABSTRAK

Analisa perbandingan proteomik *Salmonella enterica* serovar Typhi (*S. Typhi*) dan *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) menunjukkan sekumpulan protein yang diekspres unik terhadap *S. Typhi*. Salah satu daripada protein tersebut adalah hemolisin E (HlyE). Gen *hlyE* ini adalah spesifik untuk perumah manusia, tiada atau berubah fungsi dalam *S. Typhimurium*. Gen *hlyE* merupakan gen yang terdapat pada 'Salmonella Pathogenecity Island (SPI) 18'. Dalam kajian ini, kehadiran dan fungsi gen *hlyE* di dalam isolat klinikal *S. Typhi* telah ditentukan. Analisis PCR dan jujukan DNA menunjukkan bahawa *S. Typhi* mempunyai keseluruhan salinan jujukan bagi gen *hlyE*. Walaupun gen *hlyE* hadir di dalam isolat *S. Typhi*, bakteria ini tidak menunjukkan zon hemolitik pada agar darah kuda. Peningkatan aktiviti hemolitik telah dilihat apabila *S. Typhi* dikultur pada agar darah kuda menggunakan asai cakera-difusi dengan kehadiran ampisilin. Protein periplasmik yang diekstrak daripada *S. Typhimurium* dan *S. Typhi* (strain vaksin dan isolat klinikal) menunjukkan kehadiran protein 34 kDa iaitu merupakan anggaran saiz untuk HlyE. Ekstrak protein periplasmik daripada *S. Typhi* menunjukkan aktiviti hemolitik yang kuat pada agar darah. Protein rekombinan HlyE menunjukkan reaksi positif terhadap IgG sera pesakit demam kepialu, ini menunjukkan penghasilan HlyE yang reaktif ketika infeksi. Protein rekombinan HlyE yang dituliskan ini mempunyai kesan sitotoksik terhadap sel U937 manusia. Kajian ini memberikan informasi asas

terhadap aktiviti gen *hlyE* dan pengetahuan tambahan dalam kajian kepatogenan *S.*

Typhi terhadap manusia

**ISOLATION AND EXPRESSION OF HEMOLYSIN E (HlyE)
FROM *Salmonella enterica* serovar Typhi (*S. Typhi*) ISOLATES**

ABSTRACT

Comparative proteomic analysis of *Salmonella enterica* serovar Typhi (*S. Typhi*) and *Salmonella enterica* serovar Typhimurium (*S. Typhimurium*) revealed a subset of highly expressed proteins unique to *S. Typhi*. One of these proteins is hemolysin E (HlyE). The *hlyE* gene is necessary for human-host specificity, which is absent or functionally altered in *S. Typhimurium*. *hlyE* gene belongs to *Salmonella* Pathogenicity Island (SPI) 18. In this study, the presence and the function of *hlyE* gene in *S. Typhi* clinical isolates was determined. PCR and DNA sequence analyses showed that *S. Typhi*, serovar that is specific for human, harbors an intact copy of *hlyE* gene. Despite the presence of *hlyE* gene in *S. Typhi* isolates, the bacteria did not display haemolytic activity when cultured on horse blood agar plates. Increased haemolytic activity was observed when *S. Typhi* was grown on horse agar plates in disk-diffusion assay in the presence of ampicillin disk. Crude periplasmic proteins extracted from *S. Typhimurium* and *S. Typhi* (vaccine strain and clinical isolates) showed the presence of 34 kDa proteins, which is an expected size for HlyE. The crude protein extracts from *S. Typhi* showed strong haemolytic activity on the blood agar plates. HlyE recombinant protein showed positive reaction against IgG of sera from typhoid patients, suggesting reactive HlyE production during infection. The purified HlyE recombinant proteins have a cytotoxic activity towards U937 human monocytic cell line. This research provides fundamental information on the activity

of *hlyE* gene product and contributes additional knowledge in the study of *S. Typhi* pathogenesis in human.

CHAPTER 1

INTRODUCTION

1.1 *Salmonella* and disease: An overview

‘*Salmonella*’ was named after Daniel Elmer Salmon, an American veterinary pathologist, who discovered *Salmonella* bacterium isolated from pigs in 1885. Currently, there are 2579 classified serovars of *Salmonella enterica* (Grimont & Patrick, 2007) which are differentiated by their antigenic characteristics and host range (Grimont & Patrick, 2007; Popoff & Minor, 1997). The nomenclature system used for the genus *Salmonella* is based on recommendations from the Centers for Disease Control and Prevention (CDC), USA (Brenner *et al.*, 2000). The complete names of these serovars are commonly abbreviated such as ‘*Salmonella enterica* serovar Typhi’ is commonly referred as *Salmonella* Typhi or *S. Typhi* (Judicial, 2005). The various *Salmonella enterica* serovars infect a wide range of vertebrate animals. Several serovars have very narrow and restricted host. For example, humans are host-specific for serovar Typhi whereas serovar Pullorum exclusively infects chicken. These host-specific serovars cause a systemic illness that could be fatal if left untreated. In contrast to *S. Typhi*, *S. Typhimurium*, infects a wide range of hosts including humans and other vertebrates.

Infections of *S. enterica* in human may display different clinical manifestation depending on the *Salmonella enterica* serovars. There are two main clinical manifestation associated with *Salmonella* infections in humans. First, *S. Typhi* and *S.*

Paratyphi cause systemic infection called typhoid fever. This enteric fever is a life-threatening disease and display several symptoms like prolonged fever (38°C and above) followed by malaise, anorexia and nausea. 1–3% of hospitalized patients faced the more serious complication, which is intestinal perforation. After traversing the intestinal mucosa, the causative bacteria then disseminate, resulting in secondary infections of the liver, spleen, bone marrow, gallbladder and Peyer's patches of the terminal ileum (Parry *et al.*, 2002). The second is gastrointestinal disease, the more common clinical outcome of *Salmonella* infection in humans caused by non-typhoidal *Salmonella*. This non-typhoidal salmonellosis is commonly caused by *S. Typhimurium* and *S. Enteritidis*, which results in self-limiting disease such as nausea, vomiting, abdominal cramping and diarrhea. The non-typhoidal *Salmonella* are primarily transmitted to human directly or indirectly from animal sources and mostly via foodborne. In some cases the sources of this infection are pets, direct personal contact, nosocomial infection, waterborne and contaminated drugs and solutions (Hohman, 2001; Ohl & Miller, 2001). Patients less than 3 months old, greater than 50 years old, and patients with diabetes, malignancy, rheumatologic disorders, HIV infection and immunosuppression, are at risk of getting gastrointestinal salmonellosis. About 5% of non-typhoidal infections are associated with bacteremia (Hohman, 2001; Pang *et al.*, 1995).

1.2 Typhoid fever

Typhoid fever is a global health problem. According to World Health Organization (WHO), approximately 216 000 to 600 000 (1-4%) deaths are reported yearly from 21 million registered typhoid cases worldwide. It is estimated that 90% of the death cases occur in Asia. Ministry of Health (MOH) Malaysia reported that the annual

incidences of typhoid in Malaysia for the past 10 years (1998–2007) is below 5 cases per 100 000 population, which is classified as low endemic region for typhoid fever. Kelantan has the highest annual incidence when compared to other states in Malaysia. From April to June 2005, an outbreak occurred in Kelantan with 735 reported cases and 2 deaths (MOH, 2007).

A person is confirmed to have typhoid fever when he has a fever (38°C and above) for at least 3 days with a laboratory confirmed positive culture (blood, bone marrow or stool) for *S. Typhi* (WHO, 2003). Mode of infection is by ingestion of food or water contaminated with fecal that contains *S. Typhi*. The infection is transmitted from person to person through poor hygiene practices and sewage contamination of water supply (MOH, 2007). Acute systemic illness is characterized by prolonged fever, abdominal pain, and persistent bacteremia. About 10 to 20% of patients showed acute diarrhea symptom after ingestion of *S. Typhi*. This may last for several days. On the second week of infection, the fever continues and the patient may appear severely ill. Gastrointestinal complications, such as bleeding or perforation may occur at any time but usually during the third week of infection (Levine, 2009). Common physical symptoms for typhoid patients are prolonged fever, brown coated tongue, confusion, decreased auditory acuity and nuchal rigidity (Crum, 2003). Rose spots on abdomen and chest may develop on some patients (Levine, 2009).

1.3 *Salmonella enterica* serovar Typhi

S. Typhi is a rod-shape, Gram negative, facultative anaerobic, non-encapsulated, flagellated bacilli belonging to the family of *Enterobacteriaceae*. It is motile with peritrichous flagella. *S. Typhi* is identified in the laboratory by culturing on selective

media followed by several biochemical and serological tests. Suspected colony obtained from selective media is further distinguished from other *Salmonella* serotypes by its biochemical properties and agglutination with specific antisera. For example, on Salmonella Shigella (SS) agar, Salmonellae produce lactose non-fermenting colonies with black centres. The colony with such appearance was chosen for biochemical tests screening as described in Table 1.1. In serological test, *S. Typhi* is positive for LPS antigen O9 and O12, protein flagella antigen H-d and polysaccharide capsular antigen Vi (WHO, 2003).

Table 1.1: Biochemical tests for differentiation of *S. Typhi* from related subspecies (adapted from WHO, 2003)

Organism	Kligler's Iron Agar Test				Motility, Indole, Urea Tests			Citrate Test
	Slant	Butt	H ₂ S	Gas	Motility	Indole	Urea	
<i>S. Typhi</i>	Alkaline	Acid	Wk+	-	+	-	-	-
<i>S. Paratyphi A</i>	Alkaline	Acid	-	+	+	-	-	-
Other <i>Salmonella</i> sp.	Alkaline	Acid	V	V	+	-	-	V

‘+’ = Positive

‘-’ = Negative

Wk + = Weak positive

V = Variable result

H₂S = Hydrogen sulphide

1.4 Laboratory diagnosis of typhoid fever

1.4.1 Identification of *S. Typhi* by culture method

The definitive diagnosis of typhoid fever depends on the isolation of *S. Typhi* from blood, bone marrow or a specific anatomical lesion from typhoid patients. Bone marrow aspirate culture is the gold standard for the diagnosis of typhoid fever and is principally important for patients who have been previously treated, have a long history of illness and have been getting negative for blood culture at the recommended volume of blood (WHO, 2003). However, bone marrow aspirate samples are difficult to obtain and relatively (Bhutta, 2006). Thus, blood culture is the best option to diagnose typhoid fever, as more than 80% of typhoid patients have the causative bacteria in their blood (WHO, 2003).

1.4.2 Serological diagnostic methods

Serological diagnostic tests give a rapid diagnosis of typhoid fever compared to bacterial culture method, which requires 3 to 5 days to perform. There are several serological methods widely being used to diagnose typhoid fever. Felix-Widal test works by detecting agglutinating antibodies against O and H antigens. However, the test only gives moderate sensitivity and specificity. It produces false negative results for up to 30% of culture-positive cases of typhoid fever (WHO, 2003). Moreover, other *Salmonella* serotypes also possess the same O and H antigens similar to *S. Typhi* (WHO, 2003; Grimont & Patrick, 2007) and there are cross-reacting epitopes between *S. Typhi* with other *Enterobacteriaceae* (WHO, 2003). These factors can lead to false positive results. However, the test is still being used in many areas since it is an affordable diagnostic method (Baba *et al.*, 2013; Ley *et al.*, 2010).

TYPHIDOT® test kit is developed under USM license based on the principle of dot-blot enzyme immune-assay (Dot-EIA), for detecting IgM and IgG antibodies specific for a 50 kDa antigen of *S. Typhi* (Ismail *et al.*, 1991a). This protein is an outer membrane protein and is found to be a specific protein to *S. Typhi* (Ismail *et al.*, 1991b). Dot EIA test offers simplicity, speedity/rapidity, specificity (75%), sensitivity (95%) and cost-effective (Choo *et al.*, 1994). However, in convalescence or reinfection cases, the level of IgG is boosted by the secondary immune response, which mask the IgM. In order to increase the diagnostic accuracy, TYPHIDOT-M was later developed where the original TYPHIDOT® test was modified by inactivating total IgG in the sera sample. Inactivation of IgG removes competitive binding and allows access of the antigen to bind to the specific IgM (Bhutta & Mansurali, 1999). This test was reported to be useful in typhoid endemic areas, since it can be differentiate between convalescence and acute cases. This test was reported to have 92% sensitivity and 100% specificity in independent evaluation studies for all age groups of typhoid fever (Bhutta, 2006).

1.5 Pathogenicity of *S. Typhi*

Once the *S. Typhi* enters the host via ingestion of contaminated food or water, it passes through the stomach and the gastric acid barrier, then it reaches a site where it can invade the small intestine epithelial cells. *S. Typhi* invades the epithelium through two ways, which are by invasion of microfold (M) cells of Peyer's patches and by direct translocation across the absorptive epithelial cells. After penetration into the intestinal mucosa, the bacteria are immediately engulfed by macrophages but resist from being killed. Through these vehicles, they are able to enter mesenteric lymph nodes. The bacteria multiply until the critical microbial load is sufficient to